Single Delayed Rectifier Channels in the Membrane of Rabbit Ventricular Myocytes

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In rabbit ventricular cells, the delayed rectifier current ($I_k$) has not been extensively studied, and properties of single $I_k$ channels still need to be determined. In this study, we present data on a voltage-dependent channel in rabbit ventricular cells; the properties indicate that it is an $I_k$ channel.

Patch-clamp experiments were carried out on cell-attached and inside-out patches of rabbit ventricular cells. Single-channel currents were recorded at negative potentials as inward currents with 150 mM K+ in the pipette. Voltage-dependent channel activity was only present after the return from a depolarizing test pulse, indicating activation on depolarization. Single-channel conductance calculated from the current–voltage relation was 13.1 pS (pooled data, n=8). The shift in reversal potential of the unitary currents, determined at 150 and 300 mM K+ at the intracellular side of the membrane, showed that the channels were highly permeable to potassium ions. Increase of the duration or the amplitude of the depolarizing test pulse increased channel activity. The time constant for activation at +30 mV was 187 msec (pooled data, n=4). Half-activation potential was $-4.9 \pm 3.8$ mV (mean±SD), and the slope factor was $7.2 \pm 3.7$ mV (mean±SD). Current tails, reconstructed from averaged single-channel currents, revealed that the time course of deactivation decreased from 694±73 msec at $-80$ mV to 136±39 msec at $-110$ mV. Additional evidence that the channel was indeed an $I_k$ channel was provided by the observation that the channel was blocked by 10⁻⁷ M E-4031, a class III antiarrhythmic agent that has been shown to block a component of the macroscopic $I_k$ in guinea pig heart. (Circulation Research 1993;72:865–878)

KEY WORDS • delayed rectifier current • inward rectifier current • single channels • class III antiarrhythmic agents • E-4031 • rabbit ventricular myocytes

Several types of potassium outward currents are involved in the repolarization of the cardiac action potential. Variations in type and relative size of these potassium currents and other currents produce different action potential configurations in different regions of the heart.¹⁻⁶ In the ventricle, the main outward potassium currents are the transient outward current ($I_{to}$), the delayed rectifier current ($I_k$), and the inward rectifier current ($I_{ir}$).⁴⁻¹⁰

On the basis of their different kinetic properties, these currents take part in different phases of repolarization. $I_{to}$ is turned on almost immediately after the upstroke and is responsible for early repolarization, phase 1.¹¹ $I_k$ is turned on during phase 2, is responsible for the initiation of phase 3 repolarization, and determines in part the duration of the plateau phase, phase 2. Final repolarization is mainly carried by $I_{ir}$.

Prolongation of the ventricular action potential with a concomitant increase in refractoriness is considered to have an antiarrhythmic effect.¹²,¹³ Therefore, manipulation of potassium currents seems to be of interest for cardiac arrhythmias.¹⁴ Several class III antiarrhythmic agents (according to the Vaughan-Williams classification)¹⁵ that more or less selectively block potassium currents have been developed.¹⁶⁻¹⁹ Blockade of $I_k$, prolongs the action potential by a decrease in the repolarization rate of phase 3 with a concomitant reduction of the resting potential. However, preservation of the terminal phase of repolarization and of the resting potential is essential for normal activity of the fast sodium channel and, thereby, to maintenance of normal upstroke velocity of the action potential, which is essential for normal conduction velocity. Therefore, selective blockade of $I_k$ is of more interest, because it prolongs the action potential mainly by an increase in the duration of phase 2.

$I_k$ has been extensively studied in various cardiac preparations,⁷,²⁰⁻²⁴ and it has become evident that several delayed rectifying components exist. Sanguinetti and Jurkiewicz²⁵,²⁶ found both a slow ($I_{kS}$) and a fast ($I_{kF}$) activating delayed rectifier component in guinea pig atrial²⁵ and ventricular²⁶ cells. Boyle and Nernonne²⁷ found a rapidly activating K⁺ current in rat ventricle, and Yue and Marban²⁸ identified in guinea pig ventricle a potassium channel that is rapidly activated at plateau potentials. However, only Yue and Marban performed a single-channel study; the other studies concern macroscopic currents. The difficulty of complete isolation of this type of current, a prerequisite for correct kinetic analysis of $I_k$, presents a serious drawback of macroscopic current studies. Single-channel measurements may provide accurate analysis of the kinetics of $I_k$.  

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Studies at the single-channel level are still scarce. A delayed rectifier channel has been identified in embryonic chick heart cells,29-31 in frog atrial cells,32 and in guinea pig atrial33 and ventricular cells. Studies of I_K in rabbit sinoatrial and atrioventricular node cells35 suggest that I_K in the rabbit is rapidly activated compared with the classically described I_K. Single channels in rabbit ventricle have not yet been identified.

The aim of this study is to identify delayed rectifier channels in rabbit ventricular myocytes and to study their kinetic properties. In the present article, we demonstrate, by patch-clamp experiments with cell-attached patches and inside-out patches from rabbit ventricular myocytes, the existence of a voltage-dependent channel with kinetic properties similar to those of macroscopic delayed rectifier currents. Additional evidence that the presented voltage-dependent channel is indeed an I_K channel is supplied by the observation that this channel is blocked by E-4031, a class III antiarrhythmic agent known to block a component of the macroscopic I_K in guinea pig heart.26,36-38

Materials and Methods

Cell Isolation

Single ventricular myocytes were obtained from the hearts of New Zealand rabbits (1-2 kg) by enzymatic dissociation. The rabbits were anesthetized with 1 ml/kg Hypnorm (10 mg/ml fluanisone+0.315 mg/ml fentanyl citrate, Janssen Pharmaceutica, Tilburg, The Netherlands). The heart was rapidly removed and mounted on a Langendorff perfusion apparatus, where it was perfused with solutions in the following sequence: 1) Normal Tyrode's solution was used for 5 minutes to wash out the blood. 2) Ca^{2+}-free Tyrode's solution was used for 10 minutes. 3) Collagenase (1.43 mg/ml, type B, Boehringer-Mannheim, Mannheim, FRG) was added to Ca^{2+}-free Tyrode's solution (20 minutes). During the last 5 minutes of this period, 0.1 mg/ml protease (type XIV, Sigma Chemical Co., St. Louis, Mo.) was also added. The enzymatic solution was recirculated. 4) "Kraft-Brühe" (KB) solution was used to wash out the enzyme (5 minutes). All solutions were saturated with O_2. The temperature was maintained at 37°C. The ventricles were cut into pieces and gently agitated in a small beaker with KB solution to obtain single cells. These were kept in KB solution and stored at 4°C for later use the same day or the next day. During the electrophysiological experiments, the cells were superfused with normal Tyrode's solution (cell-attached configuration) or with intracellular solution (inside-out configuration) with a perfusion rate of 1 ml/min. Single-channel experiments were performed at room temperature; action potential measurements were performed at 33-37°C.

Solutions

Composition of the solutions was as follows: Normal Tyrode's solution contained (mM) NaCl 140, KCl 5.4, CaCl_2 1.8, MgCl_2 1.0, glucose 5.5, and HEPES 5.0, buffered with NaOH at pH 7.4. Ca^{2+}-free Tyrode's solution contained (mM) NaCl 140, KCl 5.4, MgCl_2 0.5, KH_2PO_4 1.2, glucose 5.5, and HEPES 5.0, buffered with NaOH at pH 6.9. KB solution contained (mM) KCl 85, KH_2PO_4 30, MgSO_4 5.0, glucose 20, pyruvic acid 5.0, creatine 5.0, taurine 5.0, EGTA 0.5, β-hydroxybutyric acid 5.0, succinic acid 5.0, and Na$_2$-ATP 2.0, along with 50 g/l polyvinylpyrrolidone-40, buffered with KOH at pH 6.9. Intracellular solution for reversal potential measurements contained (mM) KCl 150 or 300, MgCl_2 2.7 (0.5 free Mg^{2+}), EGTA 5.0, and K$_2$-ATP 5.0, buffered with KOH at pH 7.2. Pipette solution for single-channel recording contained (mM) potassium gluconate 140, KCl 10, and HEPES 10, buffered with KOH at pH 7.4. Pipette solution for action potential recording contained (mM) potassium gluconate 150, KCl 10, and HEPES 10, buffered with KOH at pH 7.2.

Data Recording

Action potentials and single-channel currents were recorded with a homemade patch-clamp amplifier using the patch-clamp technique.40 Patch electrodes were made of borosilicate glass on a homemade one-stage puller. The tips of the electrodes were heat-polished and, after filling with pipette solution, had a tip resistance of 3-5 MΩ.

Single delayed rectifier channel activity in cell-attached patches was elicited by depolarizing steps (duration, 500 msec, unless otherwise stated; frequency, 0.5 sec⁻¹). Activity of the channel was measured after subsequent repolarization to the resting membrane potential, analog to the whole-cell tail current. In several experiments, resting membrane potential of the ventricular cells was determined after the single-channel measurements. To get access to the cell interior, the patch membrane was ruptured by gentle suction to the patch pipette. The averaged resting membrane potential determined from 13 cells was approximately −70 mV (−69.2±4.3 mV [mean±SD], n=13). This value is approximately 10 mV less negative than values obtained under similar experimental conditions by others.41 Taking into consideration a liquid junction potential of approximately −13 mV between the patch pipette solution (mainly 140 mM potassium gluconate) and the bath solution (mainly 140 mM NaCl), the actual membrane resting potential should be approximately −83 mV. However, since in both whole-cell experiments and cell-attached patch measurements the error due to liquid junction potential is about the same, these errors are largely canceled out when patch potentials are expressed relative to the averaged resting membrane potential.

Action potentials were recorded in the whole-cell current-clamp mode and were elicited by the injection of depolarizing pulses (duration, 6 msec; amplitude, 2 nA; frequency, 0.5 sec⁻¹).

Single-channel currents, recorded from cell-attached patches and inside-out patches, and action potentials were either stored on digital audiotape by a digital tape recorder (model DTR 1200, Biologic, Claux, France) or on videotape via a PCM system (Sony PCM-501). Signals were off-line-filtered (200 Hz, low pass) with a two-pole Butterworth filter, digitized at a sampling interval of 781 μsec by an AD converter board (PCL-718, Advantech Ltd.), and stored on a hard disk of a personal computer (Acer 910) for further analysis.

Data Analysis

Corrections were applied to the raw data before the actual single-channel analysis.
Capacitive transient correction. Since no recordings without channel openings were available to correct for capacitive transients, this correction was accomplished by subtraction of an exponential function. The time constant and amplitude of the exponential function were adjusted until a flat baseline was obtained (Figures 1A–1C).

Subtraction of \( I_{K1} \) channel events. The abundant presence of \( I_{K1} \) channel events severely complicated single-channel analysis. In those cases in which ensemble-averaged currents had to be constructed, the absence of \( I_{K1} \) channel openings was essential. Experiments in which 2 mM BaCl\(_2\) was used to block \( I_{K1} \) showed that this block was voltage dependent and not complete. Immediately after return to negative potentials, short openings of \( I_{K1} \) were observed. Furthermore, this concentration also partially blocked the delayed rectifier channel. These observations forced us to use alternative ways to remove \( I_{K1} \) from the current tracings. A computer program was developed that enabled us to identify and subtract \( I_{K1} \) channel events on the basis of their relatively large unitary current amplitude (Figures 1C and 1D). This correction was only possible when the amount of overlapping events was relatively low.

Finally, probability density functions were constructed from these corrected data by software developed in our own laboratory and by the single-channel analysis program ASCD (Droogmans, Leuven, Belgium). Probabilities were calculated from these probability density functions after a fit was made with a number of Gaussian functions.

*Estimation of the degree of activation.* In whole-cell experiments, the degree of activation is determined by measurements of the peak amplitude of the tail current, which is considered to be proportional to the degree of activation. Analogous to this, to estimate the degree of activation in single-channel experiments, one may construct ensemble-averaged currents and measure peak amplitudes. To construct these ensemble-averaged currents, \( I_{K1} \) events had to be subtracted. The experiments in which the degree of activation was estimated were performed at a holding potential of \(-70\) mV. At this potential, numerous overlapping channel events were always present, and in those cases the program, which was developed to remove \( I_{K1} \) channel events, often failed. This was due to the fact that the program was not able to distinguish between one \( I_{K1} \) channel opening (closing) and the fast cumulative opening (closing) of two or more \( I_{K1} \) channels. For this reason, we had to choose an alternative way to estimate the degree of activation.

When the voltage at which current tails are measured is constant, one may expect that the time constant of current decay is also constant. Therefore, the area under the current tails is also proportional to the degree of activation. We used this approach to determine the degree of activation in single-channel experiments. The area under the tail current is given by

\[
A = \bar{n}_{\text{open}} \cdot T \cdot i
\]

where \( A \) is the area, \( \bar{n}_{\text{open}} \) is the mean number of channels open during time \( T \), and \( i \) is the single-channel current amplitude. In the experiments presented here, \( T \) was the first 1,500 msec after return to the resting membrane potential. Since \( T \) and \( i \) are constant, \( \bar{n}_{\text{open}} \) is directly proportional to \( A \) and thus to the degree of activation.

\( \bar{n}_{\text{open}} \) was derived from probability functions that consisted of several peaks. The area of each peak represents the probability of finding 0, 1, 2, \ldots, \( n \) channels simultaneously open. Areas were calculated from Gaussian functions fit through these peaks. \( \bar{n}_{\text{open}} \) was calculated in the following way:

\[
\bar{n}_{\text{open}} = 1 \cdot P_1 + 2 \cdot P_2 + \ldots + n \cdot P_n = \sum n \cdot P_n
\]

where \( n \) is the number of channels simultaneously in the open state and \( P_n \) is the probability of finding \( n \) channels in the open state simultaneously.

Fits for the time course of activation (Figure 6) and for the voltage dependence of activation (Figure 8) were calculated by the Marquardt least-squares method of nonlinear regression analysis\(^3\) after normalizing \( \bar{n}_{\text{open}} \) to its maximum value (\( \bar{n}_{\text{open,max}} \)) within individual experiments.
Results
Depolarization Activates a Voltage-Dependent Potassium Channel

Measurement of outward currents through the single delayed rectifier channel in mammalian cardiac cells has proven to be difficult under physiological potassium concentrations. To create optimal conditions for identifying single delayed rectifier channels, we used 150 mM extracellular potassium to increase the single-channel conductance. Even under these conditions, we were not able to detect outward single-channel currents at positive potentials. At extreme positive potentials, i.e., +100 mV, an increase in noise was observed, indicating that some channel activity was present; however, no discrete levels could be detected. For this reason, single-channel currents were measured at negative potentials as inward currents. Figure 2 shows a typical example of such an experiment in which membrane currents were recorded from a cell-attached patch of a rabbit ventricular myocyte. The patch was clamped at the resting membrane potential, and 100-mV depolarizing potential steps with a duration of 500 msec were applied intermittently. At resting membrane potential, before a depolarizing potential step (Figure 2A), a channel was observed with long openings and short closures. This channel was present in most patches and was identified as an inward rectifier potassium channel by its high open probability of 0.95 and a slope conductance of 42.8 pS (Figure 3), which is close to the value found in rabbit ventricular muscle.41,44 During the depolarizing step, no channel openings could be observed. However, after return to the resting membrane potential from this depolarizing step, a second type of channel activity was present, superimposed on inward rectifier channel openings (Figure 2B). This type of channel activity was characterized by a series of short openings that finally disappeared again, although channel activity often persisted for more than 1 second. During the first 1,500 msec after return to the membrane potential, this channel was open 20% of the time.

The unitary current amplitude of the channel was approximately one third that of the inward rectifier channel. Apparently, this channel was activated during depolarization, and decay of channel activity due to deactivation could be seen after return to resting membrane potential. We shall refer to this channel as an I\textsubscript{k} channel.

Figure 3 shows current tracings recorded from a cell-attached patch at various potentials after a depolarizing step was applied. The unitary current amplitude of both the I\textsubscript{k} channel and the I\textsubscript{k} channel increased with hyperpolarization. At more hyperpolarized potentials, the I\textsubscript{k} channels tended to close faster after return from the depolarizing clamp pulse. The current–voltage relation of the pooled data of eight experiments revealed a slope conductance of 13.1 pS for the I\textsubscript{k} channel and a slope conductance of 42.8 pS for the inward rectifier channel. When the slope conductance for each experiment was determined separately, we yielded an average slope conductance of 13.2±2.0 pS (mean±SD, n=8) for the I\textsubscript{k} channel and 42.1±2.4 pS (mean±SD, n=7) for the I\textsubscript{k} channel. The ratio between I\textsubscript{k} channels and I\textsubscript{k} channels in these cells was estimated at 1:3.5.

To decide whether this I\textsubscript{k} channel was indeed a K\textsuperscript{+} channel, we determined the reversal potential of the channel at two different intracellular K\textsuperscript{+} concentrations. For these experiments, single-channel currents were measured from inside-out patches so that both intracellular and extracellular K\textsuperscript{+} concentrations and the transmembrane voltage were precisely known. Figure 4 shows the current–voltage relation of the I\textsubscript{k} channel determined at 150 mM and 300 mM K\textsuperscript{+} at the intracel-
lular side of the cell membrane. The reversal potential, estimated by extrapolation of the current–voltage curve, shifted 20 mV in the left direction as intracellular K+ concentration was increased from 150 to 300 mM. This reversal potential shift is close to the shift predicted by the Nernst relation for a K+-selective conductance (17 mV to the left), indicating that the channel is highly permeable to potassium ions. Increasing intracellular K+ concentration significantly increased the slope conductance from 11.5 to 16.8 pS (analysis of covariance, p<0.025).

When the K+ concentration was increased from 150 to 300 mM, the Cl− concentration was concomitantly increased from 150 to 300 mM. Under these conditions, the Cl− reversal potential will shift from +72 to +90 mV. This rules out the possibility that the investigated channel is a Cl− channel.

**Time Course of Activation**

Depolarizing clamp pulses of increasing duration were applied, and single-channel activity was measured after a return to the resting membrane potential to study the time course of activation of this I\textsubscript{K} channel (Figure 5). Single-channel currents were recorded from a cell-attached patch containing three I\textsubscript{K} channels and one I\textsubscript{K1} channel. Column 1 in Figure 5 shows samples of current tracings used to generate the probability density functions shown in column 2. In this experiment, a depolarizing clamp pulse of 50 msec (Figure 5A) did not activate any I\textsubscript{K} channels. Only I\textsubscript{K1} channel activity was observed. This resulted in two peaks in the probability density function, one peak at 0 pA corresponding to the closed I\textsubscript{K1} channel and one peak at −1.88 pA corresponding to the open I\textsubscript{K1} channel. A depolarizing clamp pulse of 100 msec (Figure 5B) resulted in I\textsubscript{K} channel activity, indicated by two extra peaks in the probability density function. The peak at −0.55 pA corresponds to one I\textsubscript{K} channel open when the I\textsubscript{K1} channel is closed, and the peak at −2.35 pA corresponds to one I\textsubscript{K} channel open when the I\textsubscript{K1} channel is open. Lengthening of the depolarizing clamp pulse to 200 msec (Figure 5C) further increased I\textsubscript{K} channel activity. Two and sometimes even three I\textsubscript{K} channels were open simultaneously, resulting in extra peaks in the probability density function. The peak at −1.10 pA corresponds to two I\textsubscript{K} channels open when the I\textsubscript{K1} channel is closed, and two

**Figure 3.** Current (i)–voltage (V) relations of the delayed rectifier channel (○) and the inward rectifier channel (●). The pooled data of eight experiments were fit by linear regression, resulting in a slope of 13.1 pS for the I\textsubscript{K} channel and a slope of 42.8 pS for the I\textsubscript{K1} channel. On the right are single-channel current tracings recorded from a cell-attached patch from one of the experiments to generate the i–V plot. The patch membrane was clamped at +30 mV for 500 msec and subsequently hyperpolarized to various potentials (V1). Channel openings are seen as downward deflections.

**Figure 4.** Graph showing the current (i)–voltage (V) relation of the delayed rectifier channel at 150 mM intracellular K+ (○) and at 300 mM intracellular K+ (+). Single-channel currents, used to generate the i–V relation, were recorded from inside-out patches. The pipette contained 150 mM K+. The patch membrane was clamped at +40 mV for 500 msec and was subsequently hyperpolarized to various potentials. The pooled data of three experiments were fit by linear regression. The slope conductance was 11.5 pS at 150 mM intracellular K+ and 16.8 pS at 300 mM intracellular K+.
peaks at -2.86 and -3.33 pA correspond to two and three open I_K channels, respectively, when the I_K channel is open. Depolarizing clamp pulses of 400 msec (Figure 5D) showed a further increase in I_K channel activity seen as an increase in the area under the peaks in the probability density function. The maximum number of channels in the open state at the same time, however, remained three. From this, we conclude that there were at least three I_K channels in the patch. Channel activity did not increase after longer depolarizing clamp pulses (800 msec). This indicates that in this patch the current is fully activated within 400 msec. This type of experiment was repeated in three other cells. As a measure for the degree of activation during depolarization, \( \bar{n}_{\text{open}} \) (see “Materials and Methods”) during the first 1.5 seconds after a return to the resting membrane potential was calculated from the probability density functions. For each experiment, these values were normalized by taking \( \bar{n}_{\text{open,max}} \) as unity and were plotted versus duration of the depolarization. Figure 6 shows the pooled data of these four experiments. The data points could be fit with a single exponential function of the following form:

\[ \bar{n}_{\text{open}} / \bar{n}_{\text{open,max}} = C(1 - e^{-t/\tau}) \]

where C is the maximum value of the exponential function, t is the duration of the depolarizing step, and \( \tau \) is the time constant of activation at this potential (+30 mV). For \( \tau \), a value of 187 msec was calculated. In each experiment, \( \bar{n}_{\text{open,max}} \) was reached within 500 msec, indi-
cating that in all patches the current was fully activated within 500 msec. Furthermore, we observed in each experiment a slight decrease in $n_{\text{open}}$ on further lengthening of the depolarizing clamp pulse after reaching its maximum value. This decrease in current only becomes manifest at depolarizations longer than 500 msec and suggests that a slow inactivation process might be involved.

**Voltage Dependence of Activation**

We examined the voltage dependence of activation by applying increasing positive potentials in the cell-attached patch mode. Column 1 in Figure 7 shows samples of current tracings used to generate the probability density functions shown in column 2. A depolarizing clamp pulse from $-70$ to $-40$ mV did not induce any $I_K$ channel activity, indicating that the threshold for activation was not yet reached (Figure 7A). The probability density function shows two peaks corresponding to a closed $I_K$ channel (at 0 pA) and an open $I_K$ channel (at $-1.92$ pA). Depolarization to $-10$ mV was apparently beyond the threshold of activation. All tracings now contained $I_K$ channel openings, resulting in four extra peaks in the probability density function (Figure 7B).

**FIGURE 6.** Time course of activation. $n_{\text{open}}$, Mean number of channels open; $n_{\text{open,max}}$, maximum value of $n_{\text{open}}$. The normalized $n_{\text{open}}$ during the first 1,500 msec after return to $-70$ mV is plotted against the duration of the depolarizing pulse. The solid line represents the best fit of a single exponential function to the pooled data of four experiments. Different symbols indicate different experiments. The time constant of activation was 187 msec. The fit was allowed to approach the asymptote with a self-chosen maximum (0.86).

**FIGURE 7.** Delayed rectifier channel activity after depolarizing steps of increasing amplitude. Top panel: Voltage protocol with increasing potential ($V_1$; duration, 500 msec). Column 1: Single-channel currents from a cell-attached patch (same as in Figure 6) at $-70$ mV recorded after depolarizing steps of increasing amplitude ($V_1$) were applied. Channel openings are seen as downward deflections. Closed and unitary current amplitude levels of the delayed rectifier channel are indicated by solid lines. Column 2: Next to the current tracings, probability density functions (pdfs) are constructed from $n$ tracings (see below) of 1.5 seconds in duration. The pdf is represented on the ordinate. Panel A: Tracings at $V_1=-40$ mV ($n=43$). Panel B: Tracings at $V_1=-10$ mV ($n=44$). Panel C: Tracings at $V_1=+20$ mV ($n=42$).
One peak at −0.51 pA corresponds to one \( I_k \) channel open when the \( I_{ki} \) channel is closed, one peak at −1.01 pA corresponds to two \( I_k \) channels open when the \( I_{ki} \) channel is closed, one peak at −2.39 pA corresponds to one \( I_k \) channel open when the \( I_{ki} \) channel is also open, and one peak at −2.85 pA corresponds to two \( I_k \) channels open when the \( I_{ki} \) channel is also open. The maximum number of simultaneously open \( I_k \) channels was two. On depolarization to +20 mV, \( I_k \) channel activity further increased (Figure 7C). The maximum number of simultaneously open \( I_k \) channels increased to three, as can be seen by the three levels in the current tracings and an additional peak at −3.47 pA in the probability density function. Thus, the number of active \( I_k \) channels in the patch seen at the resting membrane potential increases when the membrane has previously been more positive. We calculated the mean number of channels open during the first 1.5 seconds after return to the resting membrane potential from probability density functions. These values were normalized by taking the maximum value of \( \tilde{n}_{op} \) as unity and were plotted versus the potential during the preceding depolarization. The data of this experiment are plotted in Figure 8. The data points were fit by a Boltzmann's equation:

\[
\tilde{n}_{op}/\tilde{n}_{op,max} = C/[1+\exp((V_h-V_m)/K)]
\]

in which \( C \) is the maximum value of the Boltzmann function, \( V_h \) is the half-activation potential, \( V_m \) is membrane voltage, and \( K \) is the slope factor. We found a \( V_h \) of −7.3 mV and a \( K \) of 7.7 mV. From this figure, it also can be seen that the threshold of activation lies at approximately −40 mV. This experiment was repeated in two other cells, and these data were also fit by a Boltzmann curve. The averaged value of \( V_h \) was −4.9±3.8 mV (mean±SD), and the averaged value of \( K \) was 7.2±3.7 mV (mean±SD).

**Voltage Dependence of Deactivation**

It has been shown that the time course of deactivation of the macroscopic \( I_k \) becomes faster at hyperpolarization of the cell membrane. To investigate whether the present \( I_k \) channel also displays this phenomenon, cell-attached patches were depolarized for 500 msec, and \( I_k \) channel activity was measured after a subsequent return to various potentials negative to the resting membrane potential. Ensemble-averaged currents were constructed from the tracings with single-channel events (Figure 9). The single-channel currents show that the \( I_k \) channels close sooner after the return from depolarization to more negative potentials. This is reflected in the faster decay of the ensemble-averaged current at more negative potentials. The current decay of the ensemble-averaged current could be fit with a single exponential function. The resulting time constants of deactivation are listed in Figure 9. The same experiment was carried out in three other cells. The averaged time constants of these four experiments are as follows: 694±73 msec for patch potential \( V_1 = -80 \) mV, 469±215 msec for \( V_1 = -90 \) mV, 212±68 msec for \( V_1 = -100 \) mV, and 136±39 msec for \( V_1 = -110 \) mV.

In all ensemble-averaged currents, a “hook” was observed, seen as a fast increase in current before current decay (inset at −100 mV). The same was found for \( I_k \) in rabbit sinus node and was thought to be the result of fast recovery of an inactivation process.\(^{35}\)

**E-4031 Blocks the \( I_k \) Channel but Leaves the \( I_{ki} \) Channel Activity Unaffected**

Ionic channels may be identified by their kinetics and ion transfer properties but also by their pharmacological properties. The class III antiarrhythmic agent E-4031 specifically blocks the \( I_k \) current.\(^{56}\) We examined the effect of this agent on the channel. Channel activity was recorded at resting membrane potential after return from a 100-mV depolarizing step before, in the presence of, and after washout of \( 10^{-7} \) M E-4031. Figure 10 shows current tracings recorded under control conditions from a patch with two \( I_k \) channels (Figure 10A). During the first 1,500 msec after return to the membrane potential, the channels were open 23% of the time. After 2 minutes of superfusion with \( 10^{-7} \) M E-4031, channel activity began to diminish (Figure 10B) and disappeared completely during the next minute (Figure 10C). The probability density function (Figure 10B) shows that when E-4031 is added, open probability decreases but that the unitary current amplitude remains unchanged. During washout, \( I_k \) channel activity gradually reappeared (Figure 10D). E-4031 in a concentration of \( 10^{-6} \) M was added to another 12 cells, and in all cases, \( I_k \) channel activity was blocked. In four cells, this effect was reversible.

Figure 11 shows a similar experiment with \( 10^{-6} \) M E-4031. This patch contained three \( I_k \) channels and one \( I_{ki} \) channel. Under control conditions (Figure 11A), the \( I_k \) channels were open 16% of the time, whereas the steady-state open probability for the \( I_{ki} \) channel was 0.89. Within the first minute after perfusion with \( 10^{-6} \) M E-4031, \( I_k \) channel activity was substantially diminished (Figure 11B) and disappeared completely after 2 minutes of perfusion (Figure 11C). The open probability of the \( I_{ki} \) channel, however, was not affected in the presence of this high concentration. The probability density function under control conditions (Figure 11A), compared with that in the presence of E-4031 (Figure 11C), shows that the unitary current amplitude of the \( I_{ki} \)
channel also was not changed. Washout of $10^{-6}$ M E-4031 (Figure 11D) did not restore $I_K$ channel activity within the duration of the experiment (10 minutes). The same concentration of E-4031 was added to another four cells, and in all cases, $I_K$ channel activity was completely blocked while open probability and unitary current amplitude of $I_K$ were unaffected. At this concentration, however, it was not possible to restore $I_K$ channel activity after washout of the drug.

E-4031 Prolongs the Action Potential

Since part of the repolarization of the ventricular action potential is carried by $I_K$, it is to be expected that blockade of this channel is reflected in prolongation of the action potential. Figure 12 shows representative action potentials recorded from a rabbit ventricular myocyte before and during exposure to $10^{-6}$ M E-4031. From the average of 20 consecutive action potentials, recorded under control conditions and recorded in the presence of $10^{-6}$ M E-4031, the duration at 90% of the repolarization (APD$_{90}$) was calculated. APD$_{90}$ increased from 367±9 msec (mean±SEM, $n=3$) under control conditions to 476±13 msec (mean±SEM, $n=3$) in the presence of $10^{-6}$ M E-4031, which is a significant increase of 30%. The increase in action potential duration is clearly caused by a prolongation of phase 2 only. After washout of E-4031, action potential duration returned to control values again (not shown).

Discussion

In the present article, we show the presence of a voltage-dependent channel in the membrane of rabbit ventricular myocytes. We conclude that this channel is an $I_K$ channel for several reasons discussed below.

Ion Transfer Properties

We measured a single-channel conductance of 13.1 pS, which compares well with the value of 11.1 pS for $I_K$ channels in rabbit sinoatrial node cells assessed under similar conditions. Only a few studies on the conductance of the delayed rectifier channel in the heart permit comparison with other mammalian species. A delayed rectifier channel with a chord conductance of 5.4 pS has been described in guinea pig ventricle. This much lower value is probably due to the physiological extracellular potassium concentration in these experiments (5.4 mM). A time- and voltage-dependent macroscopic current has been studied in isolated membrane patches of guinea pig ventricular cells. Single-channel activity could not be resolved, but it was concluded that the conductance of the single channel has an upper limit of 1 pS (extracellular potassium, 4.8 mM). In guinea pig atrial cells, two types of delayed rectifier channels have been observed: a small and a large conductance channel (extracellular potassium, 150 mM). The conductance of the large conductance channel (10 pS) compares with the conductance of the $I_K$ channel in this study. Single-channel conductances 'de-
FIGURE 10. Tracings showing that class III antiarrhythmic agent E-4031 reversibly blocks the delayed rectifier current \((I_K)\). Left column: Single-channel currents recorded from a cell-attached patch at resting membrane potential after a 100-mV depolarizing step (duration, 500 msec). This patch contained two \(I_K\) channels and no inward rectifier channels. Channel openings are seen as downward deflections. Right column: Probability density functions (pdfs) constructed from \(n\) (see below) tracings of 1.5 seconds in duration. The pdf is represented on the ordinate. Panel A: Control tracings \((n=48;\) unitary current of \(I_K\), \(-0.52\ \text{pA})\). Panel B: Tracings after 2-minute perfusion with \(10^{-7}\ \text{M}\ E-4031\) \((n=35;\) unitary current of \(I_K\), \(-0.56\ \text{pA})\). Panel C: Tracings after 3-minute perfusion with \(10^{-7}\ \text{M}\ E-4031\) \((n=34). All I_K channel activity is blocked. Panel D: Tracings after washout of \(10^{-7}\ \text{M}\ E-4031\) \((n=51;\) unitary current of \(I_K\), \(-0.54\ \text{pA}).\)

scribed for nonmammalian heart cells are substantially larger than the value observed by us. Values of 15–60 and 20 pS were reported for chick embryonic ventricle and frog atrial cells, respectively.\(^{29-32}\)

The experimentally determined ratio between \(I_K\) channels and \(I_{K1}\) channels of 1:3.5 allows us to make an estimation of the total number of \(I_K\) channels per cell. In rabbit ventricular cells, the number of \(I_{K1}\) channels was
calculated to be 30–300 per 100 \( \mu m^2 \). By assuming a cylindrical cell with a cell diameter of 20 \( \mu m \) and a length of 100 \( \mu m \) (total cell surface, 6,280 \( \mu m^2 \)), this yields a lower limit of 1,884 and an upper limit of 18,840 \( I_{K_1} \) channels per cell. Taking into account a ratio of 1:3.5, the number of \( I_K \) channels per cell would be between 538 and 5,380.

The 20-mV shift in reversal potential toward negative values, observed when the intracellular potassium concentration was raised from 150 to 300 mM, indicates that the \( I_K \) channel is highly permeable to potassium ions.

**Time Course of Activation**

After a depolarizing step to +30 mV, channel activity measured at -70 mV increased when the duration of the depolarizing step was lengthened. Maximum channel activity, indicating full activation of the current, was reached within 500 msec in all four patches. The activation time constant at +30 mV was 187 msec.
(Figures 5 and 6), which is rather long compared with the activation time constants of other (e.g., calcium, sodium, and transient outward) currents activated during depolarization. This relatively slow activation is a characteristic of $I_k$.

In all patches, a slow decay in $I_k$ channel activity was observed when the patch was depolarized for more than 500 msec. This decay in current at sustained depolarizations might indicate a slow inactivation process.

**Voltage Dependence of Activation**

Characteristic for the macroscopic $I_k$ is that the degree of activation increases with depolarization. Figures 7 and 8 show that at potentials negative to $-40$ mV there is no channel activation, whereas at potentials positive to $-40$ mV channel activity increases with depolarization. The relation between $\bar{n}_{\text{open}}/\bar{n}_{\text{open,max}}$ and the voltage during depolarization could be fit with a Boltzmann curve with a $V_\text{c}$ of $-4.9\pm3.8$ mV (mean±SD) and a slope factor K of $7.2\pm3.7$ mV (mean±SD).

**Voltage Dependence of Deactivation**

Deactivation is also voltage dependent. When the patch potential was made more negative, after the depolarizing (activating) clamp step, the decay of the ensemble-averaged current became considerably faster. The averaged time constant decreased from 634 msec at $-80$ mV to 136 msec at $-110$ mV. A consistent finding is that current decay of the ensemble-averaged currents was preceded by a fast increase in current, described as a hook (Figure 9, inset at $-100$ mV). This fast increase in current probably indicates fast recovery of an inactivation process. This inactivation process during depolarization is probably different from the slow inactivation process described in "Time Course of Activation," since the time course for both processes are far apart. Slow inactivation only becomes prominent at depolarizations much longer than 500 msec, whereas recovery from inactivation is already considerable at depolarizations of this duration. Therefore, it seems that in addition to a slow inactivation process, there also is a fast inactivation process during depolarization.

The present results indicate that the kinetics of the $I_k$ channel in the rabbit are complex and that at least one open and two, but possibly three, closed states must be postulated: 1) A first closed state is that which the channel finally reaches after a return to negative potentials (the deactivated state). 2) The hook in the ensemble-averaged current on repolarization (Figure 9) suggests an additional process, best described as a fast inactivation. This fast inactivation requires the assumption of a second closed state, which may also explain the transitions seen before the deactivated state is reached. 3) Finally, the slow decay in channel activity seen after sustained depolarizations (Figure 6) suggests the presence also of a second slow inactivation process, which requires a third closed state.

**Pharmacology: E-4031**

Prolongation of the action potential in the presence of class III antiarrhythmic agent E-4031 has been ascribed to a reduction of the delayed rectifier conductance.\(^{36}\) We found that $10^{-7}$ M E-4031 completely blocks the $I_k$ channel. Blockade of the channel was partly reversible at $10^{-7}$ M but not reversible at $10^{-6}$ M E-4031. Blockade of the channel in whole-cell measurements resulted in an increase of APD$\text{in}$ by $30\pm2\%$. Voltage-clamp studies of the macroscopic $I_k$ current have revealed that E-4031 mainly reduces the tail current and reduces the time-dependent component of the outward current to a much lesser extent.\(^{26}\) We were not able to measure single $I_k$ outward currents during depolarization, but our data confirm that E-4031 blocks $I_k$ tail current at the single-channel level. We further demonstrated that the blocking action of E-4031 on the $I_k$ channel is achieved by a decrease in open probability and not by decreasing the single-channel conductance.

Finally, our results indicate that E-4031 selectively blocks $I_k$. Our single-channel data demonstrate that $I_{K1}$ is not affected by E-4031: neither open probability nor unitary current amplitude of $I_{K1}$ channels changed in the presence of E-4031. The absence of an effect on $I_{K1}$ was further supported by the observation that both resting membrane potential and phase 3 repolarization were not altered by E-4031 (Figure 12).

Substances that selectively block the delayed rectifier with minimal effect on the inward rectifier are of interest because they permit an increase in refractoriness without affecting the terminal phase of repolarization. Preservation of this latter phase is essential for normal action of the fast Na$^+$ channel and, therefore, for maintenance of normal upstroke velocity of the action potentials.

**Comparison With Other Rapidly Activating $I_k$s in Mammalian Heart**

An important feature of the $I_k$ channel described in this article is its relatively rapid activation compared...
with the classically described $I_K$. The current was fully activated within 500 msec, whereas full activation of the classically described $I_K$ takes one to several seconds. Several other reports on relatively rapidly activating $I_K$ have been published. $I_K$ in the present study shares many properties with the $I_K$ channels described by Shibasaki35 in rabbit nodal cells and with $I_K$ described by Sanguinetti and Jurkiewicz29 in guinea pig ventricle. $I_K$ in rabbit nodal cells and $I_K$ in guinea pig ventricle are both inwardly rectifying. The fact that we were unable to measure outward currents, whereas inward currents were easily measured, strongly suggests that $I_K$ in rabbit ventricle also has strong inward rectifying properties.

The decrease in channel activity in our experiments, when the patch was depolarized for more than 500 msec, is comparable to the slow decrease in current observed during sustained depolarization in nodal cells. Shibasaki35 already suggested that this slow current decay at sustained depolarization was due to slow inactivation of $I_K$. In his experiments, the overlap of another current could not be excluded, because the inactivation process was only assessed in the whole-cell configuration. Presently, we have observed the same slow decay in current at the single-channel level. Therefore, an overlap of another current can be excluded, and the slow decrease in current at sustained depolarizations thus reflects intrinsic properties of $I_K$. Furthermore, Shibasaki35 showed a hook at the beginning of the $I_K$ deactivation process that was attributed to the fast recovery of an inactivation process. We also observed such a fast increase in current before current decay in the ensemble-averaged currents on repolarization (Figure 9).

Our data do not indicate the presence of two components of $I_K$ in rabbit ventricle, as was shown for guinea pig ventricle: a slowly activating E-4031-insensitive current ($I_{Ks}$) and the rapidly activating E-4031-sensitive current ($I_{Kf}$). First, we did not find two classes of $I_K$ channels with a different sensitivity to E-4031. The agent, even at a low concentration, blocked all $I_K$ channel activity. Second, we did not observe two distinct $I_K$ channel conductances, although we cannot rule out the possibility that activity of a very small conductance channel was missed in the noise. Finally, the ensemble-averaged currents could be fit with a single exponential function, indicating that there is only one component of $I_K$ in rabbit ventricle. An important similarity between the $I_K$ channel in rabbit and $I_K$ in guinea pig is that both are specifically blocked by E-4031.

Except for a number of similarities between the $I_K$ channel in rabbit ventricle on the one side and $I_K$ in nodal cells and $I_K$ in guinea pig ventricle on the other side, there are also some differences. Time constants for activation and deactivation in rabbit ventricle appear longer than those in rabbit nodal and guinea pig ventricular cells at comparable voltages. The much lower temperature in this study (20°C) compared with the studies in guinea pig ventricle and rabbit sinoatrial node (36°C) may explain part of this difference. Kinetics of $I_K$ strongly depend on temperature with a $Q_{10}$ of 2. However, when corrections for temperature (assuming a $Q_{10}$ of 2) are made, time constants in rabbit ventricle still remain somewhat longer.

Concerning the voltage dependence of activation, there is a difference with respect to the half-maximum activation potential. It is more positive in rabbit ventricle ($-4.9$ mV in the present study) than in rabbit nodal cells ($-25.1$ mV25) and in guinea pig ventricular cells ($-21$ mV26). The slope factors of the activation curves are similar: approximately 7 mV in all preparations (the present study and References 26 and 35). We cannot rule out the possibility that at negative potentials a prepulse of 500 msec was too short to (fully) activate the channel. If this is indeed the case, it implicates that the lower part of the resulting Boltzmann curve has shifted to the right.

The channel described in the present study seems to be quite different from the rapidly activating $K^+$ currents described by Boyle and Nerbonne27 in rat ventricle and by Yue and Marban28 in guinea pig ventricle. These currents activate within approximately 10 msec and show no inward rectification properties.

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